

Products, genetic linkage and limb patterning activity of a murine *hedgehog* gene

David T. Chang¹, Alric López², Doris P. von Kessler¹, Chin Chiang¹, B. Kay Simandl², Renbin Zhao¹, Michael F. Seldin³, John F. Fallon² and Philip A. Beachy¹

¹Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205, USA

²Department of Anatomy, Neuroscience Training Program, University of Wisconsin, Madison, Wisconsin 53706, USA

³Departments of Medicine and Microbiology, Duke University Medical Center, Durham, North Carolina 27710, USA

SUMMARY

The *hedgehog* (*hh*) segmentation gene of *Drosophila melanogaster* encodes a secreted signaling protein that functions in the patterning of larval and adult structures. Using low stringency hybridization and degenerate PCR primers, we have isolated complete or partial *hh*-like sequences from a range of invertebrate species including other insects, leech and sea urchin. We have also isolated three mouse and two human DNA fragments encoding distinct *hh*-like sequences. Our studies have focused upon *Hhg-1*, a mouse gene encoding a protein with 46% amino acid identity to *hh*. The *Hhg-1* gene, which corresponds to the previously described *vhh-1* or sonic class, is expressed in the notochord, ventral neural tube, lung bud, hindgut and posterior margin of the limb bud in developing mouse embryos. By segregation analysis the *Hhg-1* gene has been localized to a region in proximal chromosome 5, where two mutations affecting mouse limb development previously

have been mapped. In *Drosophila* embryos, ubiquitous expression of the *Hhg-1* gene yields effects upon gene expression and cuticle pattern similar to those observed for the *Drosophila hh* gene. We also find that cultured quail cells transfected with a *Hhg-1* expression construct can induce digit duplications when grafted to anterior or mid-distal but not posterior borders within the developing chick limb; more proximal limb element duplications are induced exclusively by mid-distal grafts. Both in transgenic *Drosophila* embryos and in transfected quail cells, the *Hhg-1* protein product is cleaved to yield two stable fragments from a single larger precursor. The significance of *Hhg-1* genetic linkage, patterning activity and proteolytic processing in *Drosophila* and chick embryos is discussed.

Key words: mouse, *hedgehog*, genetic linkage, limb development, gene expression, *Hammertoe*, *Hemimelic extra toes*

INTRODUCTION

Experimental manipulations of vertebrate embryos have revealed the existence of organizing centers that appear to function in the patterning of adjacent structures. The dorsal blastopore lip in *Xenopus*, for example, appears to control development of the major body axis (Spemann, 1933), while the posterior margin of the limb bud or ZPA (zone of polarizing activity or polarizing region) is capable of imposing pattern upon developing limbs (Saunders and Gasseling, 1968; Wolpert, 1969). Because these and other organizing centers contribute few of the cells that constitute the actual structure being formed, patterning activity is inferred to occur through the agency of molecules secreted from the organizing center. Until recently, however, little was known about the nature and identity of these molecules.

Drosophila development has long served as a model system for the study of molecules important in vertebrate developmental processes, including secreted signaling proteins. For example, the product of the *dpp* (*decapentaplegic*) gene, a member of the TGF- β super-family of signaling molecules

which is expressed at the dorsal pole of the embryo, acts as a concentration-dependent factor capable of imposing pattern along the entire dorsal-ventral axis of the embryo (Ferguson and Anderson, 1992). The *wingless* (*wg*) segment polarity gene, a member of the *Wnt* super-family that also includes many vertebrate representatives (reviewed by Nusse and Varmus, 1992), encodes another signaling protein that acts at somewhat shorter range in segmentation and in patterning of the embryonic cuticle. Early expression of the *wg* gene in a stripe of cells bordering the parasegment boundary is required for maintenance of appropriate gene expression in an adjacent stripe of cells on the opposite side of the parasegment boundary (DiNardo et al., 1988; Martinez Arias et al., 1988); at a later stage, specification of appropriate differentiated fates depends upon expression of the *wg* product in neighboring cells (Baker, 1988; Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992).

Another *Drosophila* segment polarity gene that has been implicated as encoding a signaling molecule with an important role in patterning is *hedgehog* (*hh*). Clones of mutant cells lacking *hh* function appear to affect adjacent structures in the

Best Available Copy

EXHIBIT A

eye and cuticle of the *Drosophila* adult (Mohler, 1988; Heberlein et al., 1993; Ma et al., 1993). In the embryo, *hh* transcription is restricted to cells in a narrow stripe adjacent to and non-overlapping with the *wingless* stripe; *hh* mutations, however, affect gene expression and cuticle pattern elements in cells outside this zone of transcription (Mohler and Vani, 1992; Lee et al., 1992; Tabata et al., 1992; Tashiro et al., 1993). The notion that *hedgehog* encodes a secreted signaling molecule is also supported by other types of evidence – in vitro translated protein products can be secreted into microsomes (Lee et al., 1992) and immunostaining of *Drosophila* embryos shows that the *hh* protein is distributed in stripes that are broader than the stripes of *hh* transcription (Taylor et al., 1993; Tabata and Kornberg, 1994; von Kessler, D.V. and Beachy, P.A. unpublished observations). Molecular characterization of the *Drosophila hh* gene (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993) revealed no sequence similarities to the products of other genes, despite the fact that many segment polarity genes do have homologues in other species (see Peifer and Bejsovec, 1992 for a review). More recently, however, several groups have demonstrated the existence of *hedgehog* homologs in chick, mouse, zebrafish and rat (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994; S. C. Ekker and P. A. B., unpublished data).

Here we present evidence for broad evolutionary conservation of *hedgehog* sequences among invertebrate species. We also confirm the existence of a family of at least three mouse *hedgehog* homologues (Echelard et al., 1993) and demonstrate the existence of two new human *hedgehog* homologues. We show that *Hhg-1*, the mouse homologue which corresponds to the independently identified *vhh-1* and *sonic hedgehog* genes in the rat and the mouse (Roelink et al., 1994; Echelard et al., 1993), is expressed in the notochord, ventral neural tube, lung bud, hindgut and posterior limb bud margin in developing mouse embryos. To elucidate *Hhg-1* function, we first demonstrated that *Hhg-1* yields effects upon gene expression and cuticle pattern similar to those of the *Drosophila hh* gene when ubiquitously expressed in *Drosophila* embryos. We also found that grafts of cells expressing *Hhg-1* can impose pattern upon the developing chick limb. In both of these systems, the *Hhg-1* protein product is cleaved to yield two stable fragments from a single larger precursor. Consistent with a role in limb patterning, we mapped *Hhg-1* by segregation analysis to a region of mouse chromosome five with tight linkage to two previously mapped limb mutants. Proteolytic processing of *Hhg-1* products and their ability to function in *Drosophila* embryos as well as in vertebrate limb patterning suggests widespread conservation of the fundamental mechanisms underlying function of the *hedgehog* multi-gene family.

MATERIALS AND METHODS

Isolation of *hedgehog* homologues

Genomic clones from *Drosophila hydei* and the mosquito *Anopheles gambiae* were isolated by low-stringency screening (hybridization at 52°C, 6× SSC; washes in 2× SSC) of a *D. hydei* genomic library in the EMBL4 lambda phage vector (a gift of M. Claudia and D. Sullivan) and of an *A. gambiae* genomic library in the lambda phage vector DASH 2 (kindly provided by J. Kassis). The initial probe for this screen corresponded to positions 389–1801 (numbering according

to Lee et al., 1992), and further analysis of the *D. hydei* clone using exon-specific probes identified three hybridizing regions that corresponded to exons 1, 2 and 3 of *D. melanogaster hh*. The flour beetle (*Tribolium castaneum*; DNA a gift from Sue Brown), the leech (*Hirudo medicinalis*; DNA a gift from G. Aisemberg), the sea urchin (*Strongylocentrotus purpuratus*; DNA a gift from A. Cameron) and the mouse and human *hh*-like sequences were initially isolated by polymerase chain reaction (PCR) using primers degenerate for all possible coding combinations of the sequences underlined in Fig. 1. PCR amplifications contained from 100 ng to 2 µg genomic DNA (depending upon the genome size of the species), 2 µM of each primer, 200 µM dNTPs (Pharmacia), 1× reaction buffer (Boehringer-Mannheim) and 2.5 units Taq polymerase (Boehringer-Mannheim) in 50 µl reactions. Amplification was as follows: 94°C 5 minutes, addition of Taq polymerase at 75°C, followed by 94°C 1 minute, 52°C 1.5 minutes and 72°C 1 minute for 30 cycles and a final extension of 72°C for 5 minutes. All PCR products were cloned into pBluescript (Stratagene) prior to sequence determination. No *hh*-like sequences were obtained using DNA from *Dictyostelium* or from *C. elegans* using this approach.

Mouse clones obtained in this manner contained 144 bases of sequence between the primer ends and were labelled with [α -³²P]dATP and used for high stringency screens of mouse cDNA libraries made from whole 8.5 dpc embryonic RNA (Lee, 1990) and from 14.5 dpc embryonic brain in the λZAP vector (a gift from A. Lanahan). Several clones corresponding to *Hhg-1* were isolated and the largest, 2629 bp in length (pDTC8.0), was chosen for sequence analysis using dideoxy chain termination (Sanger et al., 1977) and Sequenase v2.0 (US Biochemicals). Compressions were resolved by using 7-deaza guanosine (US Biochemicals). Sequence analysis made use of the Geneworks 2.0 (IntelliGenetics) and MacVector 3.5 (IBI) software packages.

Analysis of RNA expression in mouse and *Drosophila* embryos

For northern blot analysis, RNA from mouse embryos and from mouse adult tissues was isolated, electrophoresed in 1.2% agarose, blotted and probed, essentially as described by Ausubel et al. (1993). The probe used was made by random hexamer primed synthesis using the pDTC8.0 insert as a template in the presence of [α -³²P]dATP. Hybridizations and washes were performed under standard high stringency conditions (Ausubel et al., 1993).

In situ hybridization to sections of mouse embryos was essentially as described Wilkinson (1992), except that [α -³³P]rUTP was substituted in place of [α -³⁵S]rUTP for riboprobe synthesis. Briefly, 7.5–10.5 dpc mouse embryos were harvested, fixed in ice-cold 4% paraformaldehyde in PBS, dehydrated through an ethanol series, cleared in xylene and embedded in paraffin. 6 µm sections were floated on a 48°C water bath, transferred to AAS (3-aminopropyltriethoxysilane, Sigma) subbed slides, dewaxed with xylene and hybridized overnight to riboprobe in the sense or antisense orientations. Slides were washed under high-stringency conditions, dipped in Kodak NTB-2 emulsion and developed after a 10 day exposure. All sections were then stained for 30 seconds with haematoxylin (Polysciences) and mounted with Permout (Fisher). Sense and antisense probes were synthesized using a riboprobe synthesis kit from Stratagene with a 249 bp *Bam*HI/*Sma*I fragment of pDTC8.0 that extends from residues 297 to 380 within the *Hhg-1* open reading frame (Fig. 1) subcloned into Bluescript as template (pDTC1.8). Adobe Photoshop was used for superimposition of bright-field and dark-field views, collected in digital form using a Sony 3 CCD camera attached to a Zeiss Axioplan microscope and transferred directly to a Macintosh Quadra 800 equipped with a NuVista Video Capture Board.

In situ hybridization to *Drosophila* embryos was performed according to standard methods (Tautz and Pfeifle, 1989). The *wingless* (*wg*) probe was made by random hexamer primed synthesis (Feinberg and Vogelstein, 1983) using a 2.2 kb *Hind*III/*Xba*I fragment from a

wg cDNA (gift from R. Nusse; Rijsewijk et al., 1987) as template. Probe synthesis was carried out in the presence of digoxigenin-dUTP (Boehringer Mannheim).

Drosophila germ-line transformation and phenotypic analysis

The *hshh* construct was made by inserting a blunted 1581 bp *MseI* fragment containing the full *hh* ORF (from 327 to 1908, Lee et al., 1992) into the *StuI* site of pCaSpeR-hs (Thummel et al., 1988; from C. Thummel, University of Utah, Salt Lake City). The *hsHhg-1* construct was made by inserting a blunted 1330 bp *Bsu36I/Eco57I* fragment from pDTC8.0 that contained the entire *Hhg-1* open reading frame into the *StuI* site of pCaSpeR-hs. *hshh* and *hsHhg-1* each were coinjected with $\text{p}\pi 25.2$ wc into *w¹¹¹⁸* embryos using a standard protocol for P element-mediated transformation (Rubin and Spradling, 1982). Germ line transformants with P element integration on the third chromosome were isolated for each construct; *hshh* was maintained as a homozygous stock and *hsHhg-1* was maintained over the TM3 balancer chromosome.

Embryos for cuticle analysis were collected and aged at 25°C and heat shocked for 1 hour at 37°C. After further incubation for 24 hours at 25°C, embryos were dechorionated, transferred to Hoyer's mountant (Wieschaus and Nusslein-Volhard, 1986) and incubated at 65°C for 5 hours. For in situ hybridization, *Drosophila* embryos from the *hs-hh*, *hs-Hhg-1* and *w¹¹¹⁸* parent lines were collected for 5 hours at 25°C, aged an additional 5 hours at 25°C, heat shocked for 1 hour at 37°C and allowed to recover at 25°C for an additional hour before fixation.

Chick limb patterning assays

Isolation and characterization of the quail cell line QT6 has been described (Moscovici et al., 1977). QT6 cells were cultured on 3.5 cm uncoated plastic culture dishes (Falcon) in growth medium (M199 medium plus Earle's balanced salt solution [Gibco, Grand Island, NY] supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, 1% dimethylsulfoxide, 100 U/ml of penicillin and 100 µg/ml of streptomycin) in a 5% CO₂ atmosphere.

QT6 cells were transiently transfected by a modified calcium phosphate method (Chen and Okayama, 1987). In brief, after preincubation in transfection medium (DMEM plus 5% fetal calf serum + 1% DMSO) 20–25 µg of precipitated DNA was added to 70–80% confluent QT6 cells in dishes. After overnight incubation, the DNA precipitate was removed and complete growth medium added. The pCIS plasmid, which carries a cytomegalovirus (CMV) promoter and SV40 intron and polyadenylation signal (Gorman, 1985), was used as the expression vector. Expression constructs included pCIS*lacZ* and pCIS*Hhg-1*, which contain *lacZ* and *Hhg-1* respectively under control of the CMV promoter. To assess transfection efficiency parallel plates were transfected with equimolar amounts of either pCIS-*lacZ* or pCIS*Hhg-1*.

For β -galactosidase activity staining, cells and limb buds were fixed 5 minutes and 1 hour, respectively, in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. After rinsing in PBS, samples were incubated in X-gal cocktail (1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl 5-D-galactopyranoside), 2 mM MgCl₂, 16 mM K₃Fe(CN)₆, 16 mM K₄Fe(CN)₆) for 18–24 hours at 22°C.

Transiently transfected QT6 cells were scraped from tissue culture plates with a Teflon scraper (Falcon) and dissociated by repeated pipetting. Poly-D-lysine (Sigma, P1149) was added to the cell suspension to a concentration of 33 µg/ml. Cells were then pelleted by centrifugation at 1×10^5 revs/minute on a benchtop microfuge for 10 seconds. Wedge-shaped fragments were excised from the pelleted cells and grafted to anterior, mid-distal, or posterior slits made with fine forceps in stage 20–21 chick wing buds (Riley et al., 1993). Embryos harvested at day 10 were fixed overnight in 10% formaldehyde, stained with Victoria blue and cleared in methyl salicylate (see Riley et al., 1993).

Detection of *Hhg-1* protein

Region-specific antibodies were generated by immunization of New Zealand White rabbits with PCR-generated, His₆-tagged fusions (in the vector pTrcHis from InVitrogen, San Diego, CA) to residues 25–159 (N-terminal) and 202–389 (C-terminal) of the *Hhg-1* ORF (Fig. 1). Following repeated boosts, reactive sera were purified using affinity matrices carrying fusions of glutathione-S-transferase to the same portions of the *Hhg-1* ORF (in the vector pGEX from Amrad, Melbourne, Australia). Specific antibodies were eluted with a buffer containing 100 mM glycine-HCl at pH 2.5 (Harlow and Lane, 1988).

For immunodetection, samples of transfected and untransfected QT6 cells and of heat-shocked wild-type and *hsHhg-1* *Drosophila* embryos were suspended and boiled in sample loading buffer and electrophoresed in 12% polyacrylamide-SDS gels (Laemmli, 1970). Following transfer to nitrocellulose (Burnette, 1981), proteins were detected by chemiluminescence (with the ECL kit from Amersham), with affinity purified anti-*Hhg-1* antibodies at a dilution of 1/300 and HRP-conjugated goat anti-rabbit 2° antibody (Jackson ImmunoResearch, Baltimore MD) at a dilution of 1/10,000.

Chromosome localization of *Hhg-1*

C3H/HeJ-*gld* and *Mus spretus* (Spain) mice and [(C3H/HeJ-*gld* × *Mus spretus*)F₁ × C3H/HeJ-*gld*] interspecific backcross mice were bred and maintained as previously described (Seldin et al., 1988). *Mus spretus* was chosen as the second parent in this cross because of the relative ease of detection of informative restriction fragment length variants (RFLV) in comparison with crosses using conventional inbred laboratory strains.

DNA isolated from mouse organs by standard techniques was digested with restriction endonucleases and 10 µg samples were electrophoresed in 0.9% agarose gels. DNA was transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH), hybridized at 65°C and washed under stringent conditions, all as previously described (Sambrook et al., 1989). Clones used as probes in the current study included a ~500 bp 3'-UTR of *Hhg-1*, a quinoid dihydropteridine reductase (Qdpr) clone, DHPRI3 (Lockyer et al., 1987) and an interleukin 6, (Il-6) specific clone, 27-4 (Mock et al., 1989).

Gene linkage was determined by segregation analysis (Green, 1981). Gene order was determined by analyzing all haplotypes and minimizing crossover frequency between all genes that were determined to be within a linkage group. This method resulted in determination of the most likely gene order (Bishop, 1985).

Characterization of *Hhg-1* sequences in *Hm* and *Hx* mutants

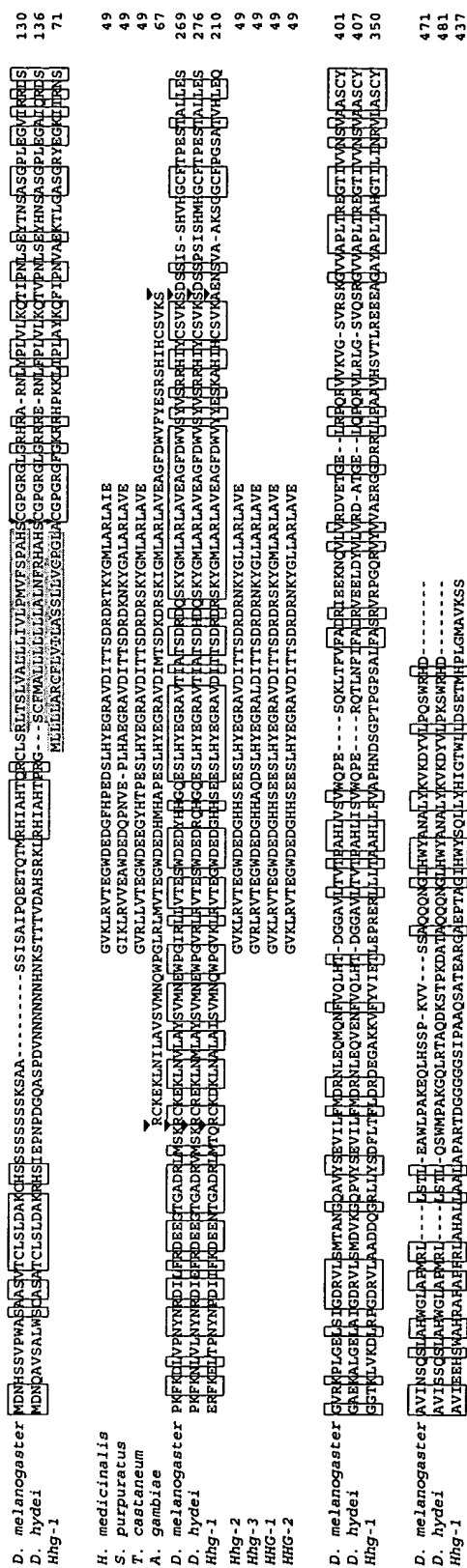
DNA from heterozygous *Hm* (AKR.C3H-*Ca Hm Sl*) and heterozygous *Hx* (B10.D2/nSn-*Hx*+) mutant individuals were obtained from Jackson Laboratory and digested with *EcoRI*, *BamHI*, *TaqI*, *HindIII*, *AluI*, *RsaI*, *DpnI*, *HinfI* and *HinPI*. These digests were electrophoresed, blotted and probed as above with ³²P-labelled pDTC8.0 and compared to similarly digested and probed DNAs from parental strains. No differences in restriction fragment lengths were detected for either mutant. This analysis would have detected differences as small as 100 bp.

Hhg-1 coding sequences were isolated by PCR amplification from genomic DNA of individuals heterozygous for the *Hx* mutation (B10.D2/nSn-*Hx*+/+; Jackson Labs). Analysis included eleven independent clones representing coding sequences from exon one, fourteen independent clones representing coding sequences from exon two and eight independent clones representing coding sequences from exon three.

RESULTS

Isolation of *hedgehog* homologues

As a first step toward isolation of *hedgehog* homologues from distant species, we used low-stringency hybridization to isolate



genomic *hh* clones from two other dipterans, *Drosophila hydei* and the mosquito *Anopheles gambiae*. We then used the polymerase chain reaction (PCR) with degenerate primers from conserved regions within the second exon (underlined regions in Fig. 1) to isolate single *hh*-like sequences from genomic DNA of the flour beetle, leech and sea urchin, and multiple sequences from mouse and man. No *hh*-like sequences were obtained using DNA from *Dictyostelium* or from *C. elegans* by this approach. From sequence comparisons, human PCR fragments 1 and 2 appear to correspond most closely to mouse fragments 1 and 2, respectively.

Our focus here is primarily upon one of the three mouse clones, *Hhg-1*, which when used as a probe yielded a 2.0 kb clone from a 8.5 dpc mouse embryonic cDNA library and a 2.7 kb clone from a 14.5 dpc embryonic cDNA library. The 2.7 kb cDNA appears to represent a nearly full-length mRNA because it corresponds to a 2.8 kb band detected by hybridization on a northern blot (see below). The largest methionine-initiated open reading frame within this cDNA encompasses 437 codons and is preceded by one in frame upstream stop codon (not shown). Sequence comparisons indicate that the protein encoded by *Hhg-1* is identical to the independently characterized mouse *Shh* (Echelard et al., 1993) except for an arginine to lysine difference at residue 122. *Hhg-1* also corresponds closely to the rat *vhh-1* gene (97% amino acid identity; Roelink et al., 1994), the chicken *Sonic hedgehog* (81% identity; Riddle et al., 1993) and *Shh* from the zebrafish (68% identity; Krauss et al., 1993; Roelink et al., 1994; S.C. Ekker and P.A.B., unpublished data). The PCR-generated fragments *Hhg-2* and *Hhg-3* appear to correspond to the *Indian* and *Desert* classes of mouse *hedgehog* genes, respectively (Echelard et al., 1993).

Alignment of the *Hhg-1* open reading frame with the two *Drosophila hh* sequences (Fig. 1) shows that all three proteins contain hydrophobic amino acid sequences near their amino-termini; the hydrophobic stretches within the *D. melanogaster* protein (residues 64 to 83) and within the mouse protein are known to act efficiently as signal sequences for cleavage (Lee et al., 1992, and J. J. Lee and P. A. B., unpublished data). Both *Drosophila* signal sequences are unusual in their internal locations, while the hydrophobic stretch of the mouse gene occurs at the extreme amino-terminus, a more conventional location for cleaved signal sequences. Although portions of

Fig. 1. Multiple mammalian and invertebrate *hedgehog*-like sequences. The *Drosophila melanogaster hedgehog* open reading frame is shown aligned with a complete *hedgehog* coding sequence deduced from genomic sequence for *Drosophila hydei* and a complete mouse coding sequence (*Hhg-1*) deduced from a cDNA clone. Amino acid identities between these complete sequences are boxed. Kyte-Doolittle hydrophobic domains are shaded, predicted signal sequence cleavage sites (von Heijne, 1986) are indicated by an arrow; and intron/exon boundaries are marked by triangles. Below these complete sequences are shown partial sequences deduced from cloned PCR products for two other mouse genes (*Hhg-2* and *Hhg-3*) and two human sequences (*HHG-1* and *HHG-2*). Sequences from invertebrate species above the complete sequence alignments include partial sequences for the mosquito *Anopheles gambiae* (from a genomic clone) and PCR-derived sequences from the flour beetle, *Tribolium castaneum*, the urchin, *Strongylocentrotus purpuratus* and the leech, *Hirudo medicinalis*. Degenerate primers used for PCR reactions incorporated sequence from the underlined portion of the *D. melanogaster* sequence.

sequence N-terminal to the *Drosophila* signal sequences are conserved, suggesting a functional role, the mouse gene lacks this region.

The overall level of amino acid identity between *Hhg-1* and *hh* carboxy-terminal to the signal sequences is 46%. A closer examination shows that the amino terminal portion, from residues 25 to 187, displays 69% identity, while remaining residues in the carboxy-terminal portion display a much lower 31% identity. Like *hh*, the *Hhg-1* coding sequence is divided into three exons and the boundaries of these exons are at the same positions within coding sequence as those of the three *Drosophila hh* exons (see Fig. 1). Curiously, the boundary between coding sequences of the second and third exons occurs near the transition from high to low levels of overall sequence conservation. The coincidence of these two boundaries suggests a possible demarcation of functional domains within these proteins. This location within *Hhg-1* coding sequence

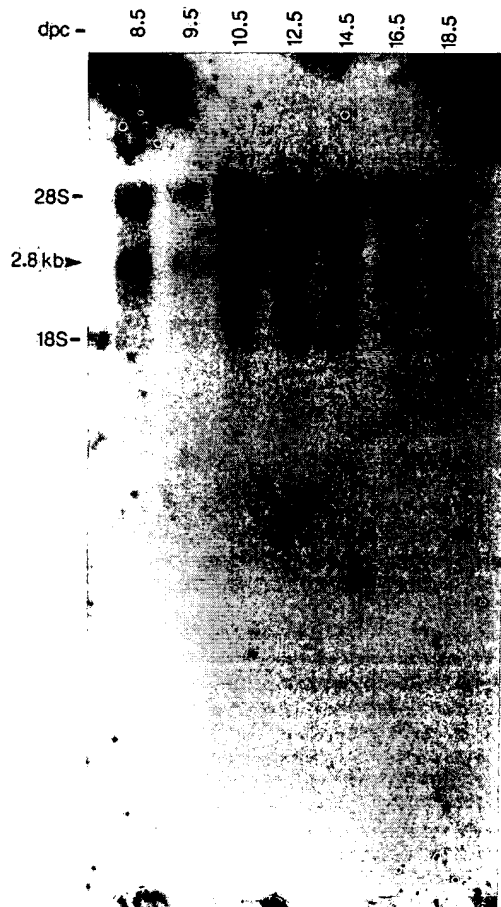


Fig. 2. Electrophoretic analysis of *Hhg-1* RNA. Each lane contains 10 µg total RNA from mouse embryos staged as indicated above the lanes (dpc, days post coitum). The probe, made from the full length *Hhg-1* cDNA, detected a ~2.8 kb band (indicated by arrowhead) in RNA from all stages of embryos examined. The upper band comigrates with the 28S RNA and is due to non-specific hybridization.

also coincides approximately with the site of a presumed proteolytic cleavage (see below).

Expression of *Hhg-1* in mouse embryos

We began our analysis of *Hhg-1* expression by hybridization of a 32 P-labelled *Hhg-1* probe to a northern blot of RNA isolated from embryos ranging from 8.5 to 18.5 dpc. A band of ~2.8 kb was detected at each stage, with a peak at day 10.5 (Fig. 2). These results are similar to those reported by Echelard et al. (1993) for *Shh* except that we detect the 2.8 kb RNA throughout embryogenesis. To obtain more detailed spatial and temporal information regarding *Hhg-1* expression, sections from 7.5, 8.5, 9.5 and 10.5 dpc embryos were hybridized to a 33 P-labelled antisense RNA probe under stringent hybridization and wash conditions (see Materials and methods); the corresponding sense RNA probe was used as a control. Selected sections from these in situ hybridizations are presented in Figs 3-5 and described below.

In the 7.5 dpc embryo, *Hhg-1* expression is confined to anterior midline mesoderm. No expression is seen in the overlying ectoderm (Fig. 3B,C) or in other embryonic or extraembryonic tissue (data not shown). Transverse sections confirm restriction of expression in the early gastrula to axial mesoderm (Fig. 3D-F); this mesodermal expression extends caudally with retraction of the node and is maintained through to formation of the notochord by 8.5 dpc (data not shown).

At 9.5 dpc, well after neural tube formation, strong expression of *Hhg-1* is seen in the entire notochord and in the ventral midline of caudal portions of the neural tube. More rostrally within the neural tube, *Hhg-1* expression extends ventrolaterally to encompass ~40% of the ventral neural tube at its maximum extent in the midbrain. Even more rostrally in the midbrain, midline expression is lost but reappears in a portion of the diencephalon (Fig. 4B). Horizontal sections demonstrate that expression rostral to the midbrain (Fig. 4C) splits and extends bilaterally (Fig. 4D,E), finally re-uniting in the ventral

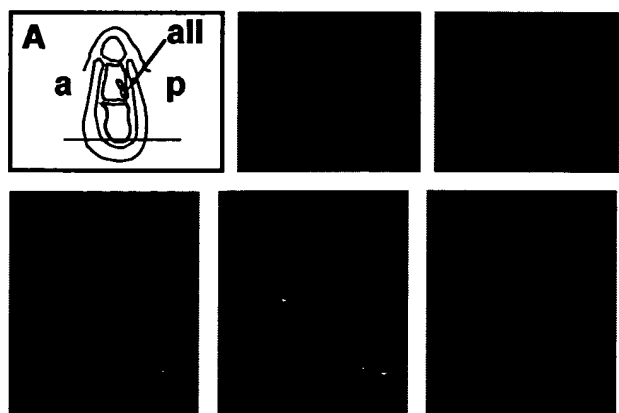


Fig. 3. *Hhg-1* expression at late gastrulation. (A) Schematic diagram showing 7.5 dpc mouse embryo, at late gastrulation. (B,C) Midsagittal sections through the egg cylinder showing hybridization in the axial mesoderm. (D-F) Adjacent horizontal sections through the egg cylinder at the level indicated in A. Note the hybridization in the midline mesoderm (asterisk). a, anterior; all, allantois; ect, ectoderm; mes, mesoderm; p, posterior.

midline of the diencephalon (Fig. 4B,E). *Hhg-1* expression thus is confined to a ring of cells in the ventral surface of the midbrain-diencephalic region. *Hhg-1* expression in the 10.5 dpc embryo is similar to that of the 9.5 dpc embryo, with strong expression in the notochord and most of the ventral neural tube and rostral neural tube expression remaining restricted to a ring of ventral cells. *Hhg-1* expression can also be observed in endoderm lining the future pharynx and foregut, with more intense expression occurring in the budding lungs; expression can also be detected in the hindgut. Finally, expression of *Hhg-1* in the limb buds at 10.5 dpc is restricted to the posterior margins of the forelimb (Fig. 5G-J) and hindlimb (data not shown). This expression clearly is restricted to the mesoderm and is absent from the overlying ectoderm, including the apical ridge. Our analysis of *Hhg-1* expression in the mouse embryo is consistent with that presented for *Shh* (Echelard et al., 1993) and for *vhh-1* in the rat embryo (Roelink et al., 1994).

Hhg-1 can function in *Drosophila* embryos

As a first step toward understanding the function of mouse *hedgehog* genes, we compared the effects of *Hhg-1* and *Drosophila hh* when ectopically expressed in *Drosophila* embryos under control of a heat inducible promoter. As described in Materials and Methods, germ-line insertions were made by P-element-mediated transformation of each gene cloned downstream of the *Drosophila hsp70* promoter. Our analysis focused on one transformant line for each construct, designated *hshh* and *hsHhg-1*. Transcription of *hh* in the *Drosophila* embryo is normally restricted to a thin stripe of cells posterior to the parasegment boundary in each segment; expression of the *wingless* (*wg*) gene is normally restricted to a thin stripe of cells anterior and immediately adjacent to the *hh* stripe. Previous studies have demonstrated a dependence upon *hh* function for the maintenance of *wingless* expression (DiNardo et al., 1988; Martinez Arias et al., 1988); the spatial restriction of *wg* expression to this thin stripe is thought to result from limited diffusion of the signal encoded by *hh*. Ectopic expression of *hh* thus would be expected to cause an expansion in the domain of *wg* expression.

As shown in Fig. 6D,E, ubiquitous expression of *hh* induced by heat shock indeed causes an expansion in the extended germ band expression domain of the *wingless* gene, as has also been demonstrated by Ingham (1993). In

addition, ectopic expression of *hh* produces consistent alterations in the size and orientation of denticles in the ventral cuticle (Fig. 6F; see Bejsovec and Wieschaus, 1993, for a description of the wild-type denticle pattern). The simplest interpretation of these changes is that bristle rows 4, 5 and 6 are replaced by bristles of size, shape and polarity normally associated with the denticles in rows 2 and 3, and our observations are again consistent with those of Ingham (1993). Neither of these changes occur in heat shocked wild-type embryos (Fig. 6A,C).

Similar analyses of ectopically expressed *Hhg-1* also reveal an expansion in the *wg* expression domain and effects upon the denticles in rows 4, 5 and 6 (Fig. 6G,I). The early effect on *wg*

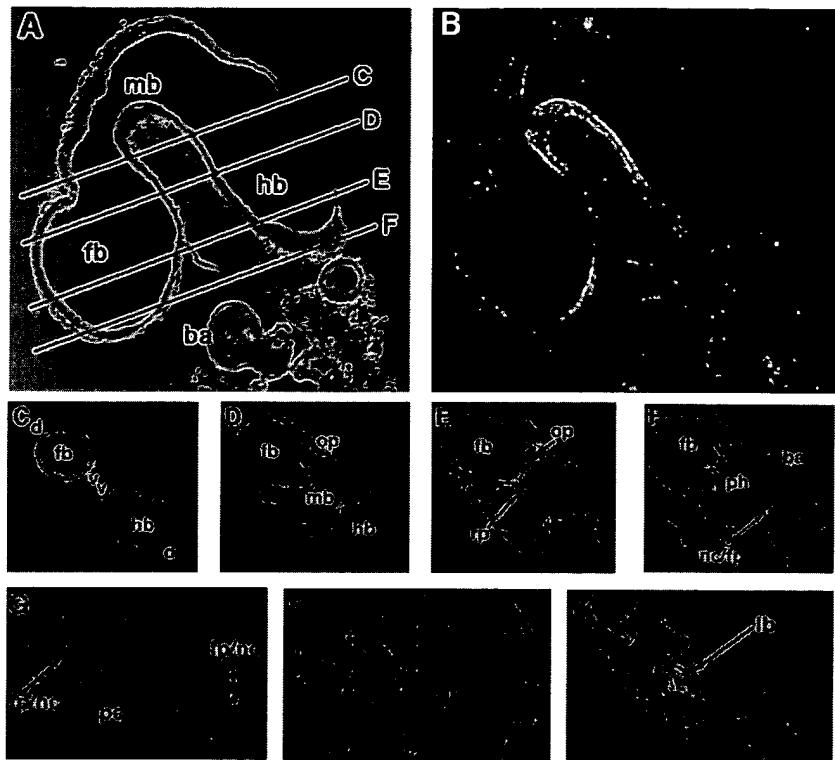


Fig. 4. *Hhg-1* expression in the 9.5 dpc embryo. (A,B) Bright and dark field views of a parasagittal section from a 9.5 dpc mouse embryo showing hybridization in the ventral midbrain and in a small patch of the ventral diencephalon. (C-F) Serial horizontal sections from superior to inferior levels in the head region of a 9.5 dpc mouse embryo. Broad, intense ventral hybridization is observed in the boundary region of the midbrain and forebrain (C). Rostrally, ventral-most expression is lost leaving two ventral/lateral domains of neural tube expression in cells adjacent to the optic vesicle. (D,E). Expression re-unites in a single midline domain of ventral neural tube cells overlying the pharyngeal lumen. Caudal to the hindbrain, neural tube expression is confined to the ventral midline (C-E) and expression is seen in the notochord beginning at its most rostral point (F). (G) Horizontal section at the level of the pericardiac region. The neural tube is cut twice in cross section at these levels and expression is likewise seen in floor plate and notochord of both cross sections. (H) Higher magnification view of G showing intense hybridization to floor plate and notochord. (I) Horizontal section at a lower level showing expression in the developing lung bud. In F, G and I, note the closer apposition of notochord to neural tube at more extreme rostral and caudal levels, indicative of an earlier stage of maturation relative to the intermediate level shown in H. ba, branchial arch; d, dorsal; fb, forebrain; fp, floor plate; lb, lung bud; mb, midbrain; nc, notochord; op, optic vesicle; pc, pericardiac region; ph, pharyngeal lumen; rp, Rathke's pouch; v, ventral.

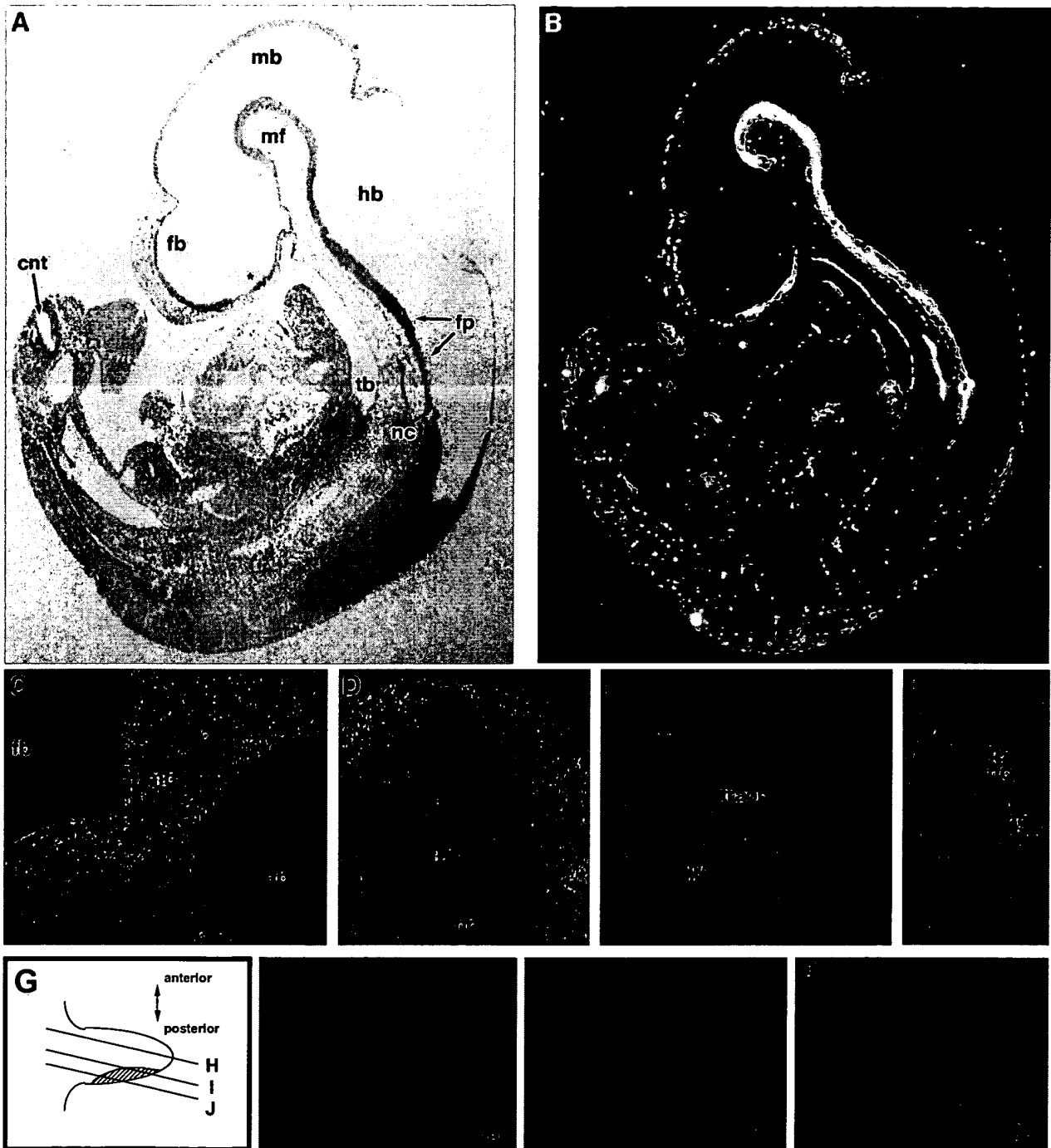


Fig. 5. *Hhgl-1* expression in the 10.5 dpc embryo. (A,B) Bright- and dark-field views of a sagittal section from a 10.5 dpc mouse embryo showing intense hybridization in the ventral neural tube and notochord, ventral diencephalic region (asterisk), and tracheal branch point. (C) Horizontal section showing hybridization in the ventral epithelium of the midbrain. (D) Horizontal section at lower level showing expression in floor plate and notochord. (E) Horizontal section showing hybridization in the epithelia of the tracheal lumen. (F) Horizontal section showing continued expression in the floor plate and notochord at caudal levels and expression in the epithelia of the hindgut. (G) Schematic diagram of developing limb, and reconstruction of expression from serial sections. Lines indicate approximate levels of sections shown in H-J. (H-J) Anterior to posterior sections of developing forelimb. Intense expression is observed in the posterior but not anterior mesoderm. No expression is observed in the apical ectodermal ridge. aer, apical ectodermal ridge; cnt, caudal neural tube; fb, forebrain; fp, floor plate; hb, hindbrain; hg, hindgut; mb, midbrain; mf, mesencephalic flexure; nc, notochord; tb, tracheal branch point; trach, tracheal lumen.

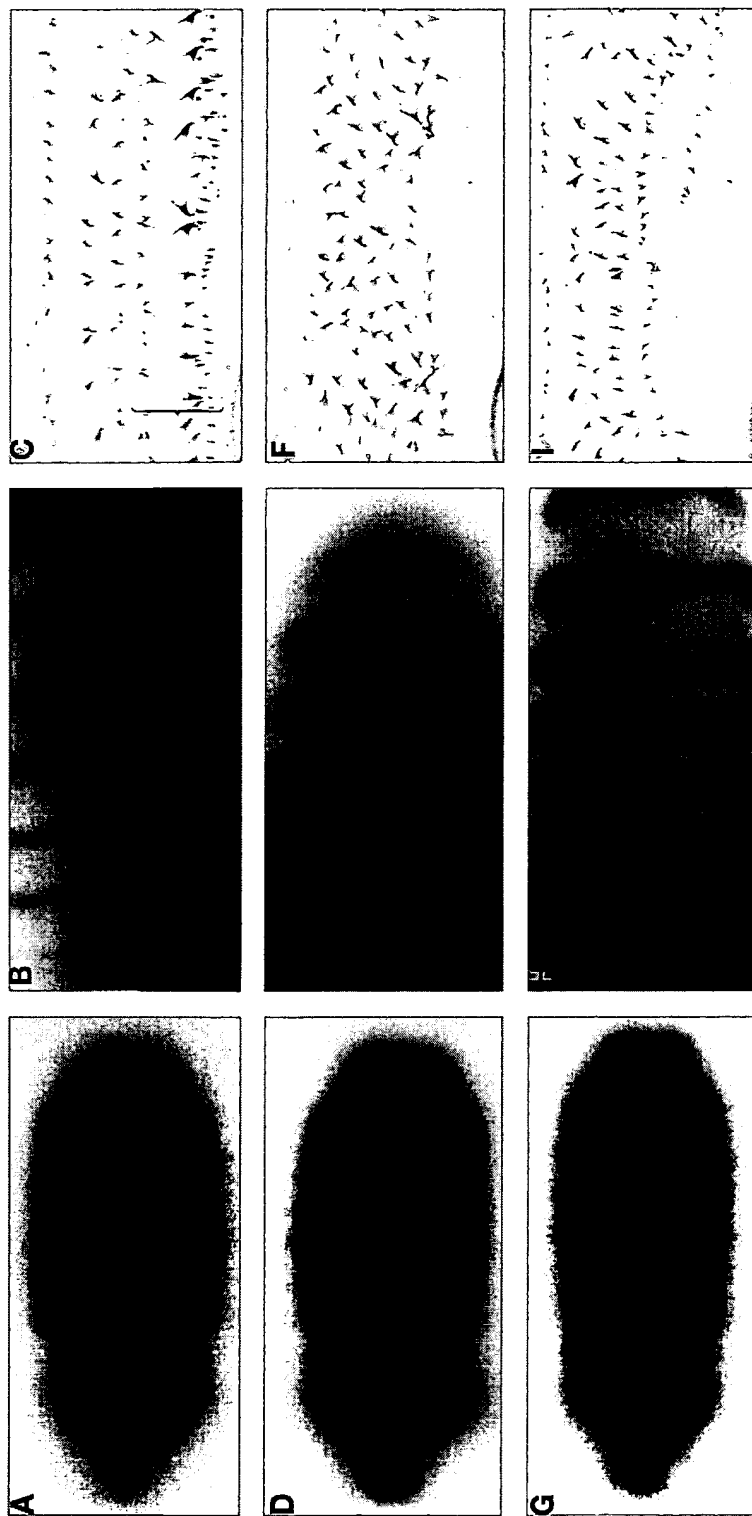


Fig. 6. Ectopic expression of *Hh*-1 in the *Drosophila* embryo. A, B, D, E, G and H show ventral views of germ-band-extended (A, D, G) and retracted (B, E, H) embryos which have been heat shocked and processed for in situ hybridization to detect *wingless* RNA expression. C, F and I show the pattern of ventral denticles within a single segment from heat-shocked embryos just prior to hatching. The genotypes of embryos in A-C are *w*^{+/18}

(wild-type control), while embryos in D-F and G-I, respectively, carry the *hshh* and *hsHh-1* construct (see text). Note that, relative to wild type (A, B), the *wingless* stripes are expanded at the extended and retracted germ band stages for embryos carrying the *hshh* (D, E) and *hsHh-1* (G, H) constructs. Note also that the wild-type polarity and character of the bristle rows 4-6 (bracketed portion of C; see text) are altered in F and I.

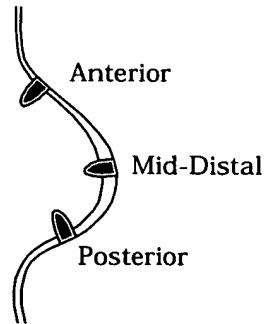


Fig. 7. Graft sites for limb patterning assays.

expression is indistinguishable from the *hh* effect. The denticles appearing in place of the posterior three denticle rows, however, appear more disorganized, with occasionally a missing denticle row and in some cases an unusual posterior row of anteriorly oriented denticles (Fig. 6I).

The patterns of *wg* expression thus far described pertain to the extended germ band stage. We also examined, however, the effects of ectopic *hh* and *Hhg-1* expression upon later stage embryos which had completed or nearly completed the process of germ band retraction. As shown in Fig. 6B,E,H, the *wg* expression domain is expanded relative to the wild type even at this later stage. The competence of cells in the expanded *wg* domain to respond to the ectopic *hh* signal at this late stage reveals a new requirement for temporal and spatial expression of candidate receptors for the *hh* signal (see Discussion).

Patterning activity of *Hhg-1* in the developing chick limb

Hhg-1 expression in mouse limb buds is restricted to mesoderm along the posterior margin of the limb bud (Fig. 5G-I), a location reminiscent of the polarizing region in the chick limb bud. Given the ability of *Hhg-1* to function in as diverged a species as *Drosophila* (see above) and in light of previous reports of chick limb patterning activity present in grafts derived from mouse limb buds (Tickle et al., 1976; Fallon and Crosby, 1977), we tested the possibility that *Hhg-1* encodes an

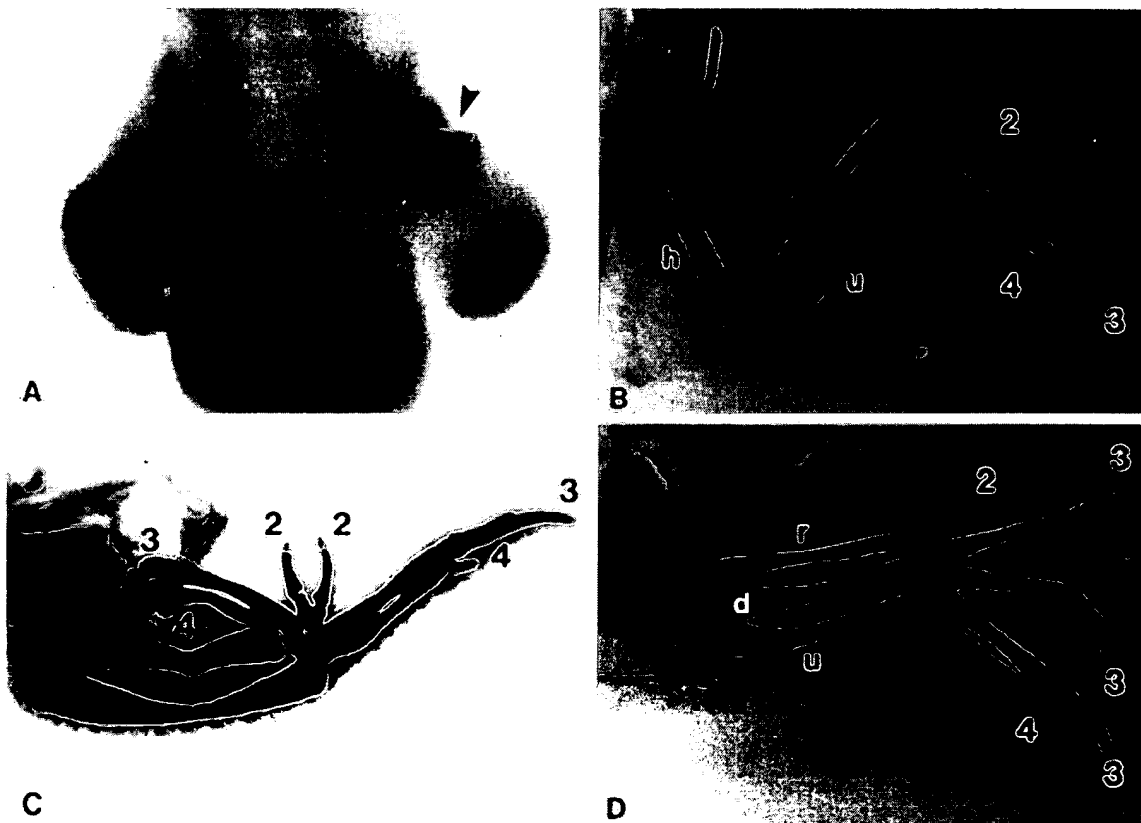


Fig. 8. Limb patterning activity of *Hhg-1*. Grafts of QT6 cells transfected with pCISlacZ (A) or pCISHhg-1 (B,C,D) were made to the anterior border (A,C) the posterior border (B) or to the mid-distal border (D) of forelimb buds within chick embryos at stage 20. The embryo in A was fixed 24 hours after grafting and stained for β -galactosidase activity (positive region indicated by arrowhead). Embryos in B-D were fixed, stained and cleared 7 days after grafting. The posterior border graft in B resulted in a normal limb skeleton (h, humerus; r, radius; u, ulna; 2,3 and 4 indicate digit identities). The anterior border graft in C caused a mirror image duplication of the manus with a digit pattern of 4-3-2-2-3-4. The mid-distal border graft in D induced skeletal duplications of digits and of the forearm: d indicates a duplicated forearm bone that probably is an ulna; the digit pattern from anterior is 2-3-3 followed by the normal 3-4.

activity capable of imposing pattern upon chick limbs. The strategy for these experiments involved high-efficiency transient transfection of the QT6 quail cell line (Moscovici et al., 1977), followed by grafting of wedge-shaped sections of transfected cell pellets to anterior, mid-distal or posterior borders of stage 20-21 chick wing buds (see Fig. 7). Initial transfections using the bacterial β -galactosidase expression gene in the vector pCIS (Gorman, 1985), which carries a cytomegalovirus promoter and an SV40 intron and polyadenylation signal, yielded expression in greater than 90% of the QT6 cells plated for transfection.

Fig. 8 shows that grafts of cells transfected with a *Hhg-1*-expression construct to anterior and mid-distal but not posterior locations within developing limb buds induced duplications of digits. Duplications induced by anterior border grafts were in mirror-image orientation relative to the normal pattern, with a typical sequence of digits shown in Fig. 8C (4-3-2-2-3-4). Mid-distal grafts commonly yielded digits in the sequence 2-3-3-4, with divergent curvature of adjacent third digits indicative of the location of the graft site (Fig. 8D). Grafts of β -galactosidase-expressing cells or posterior grafts of *Hhg-1*-expressing cells, in contrast, did not alter normal limb pattern (Fig. 8A,B). With respect to digit duplications and polarity, all grafts of *Hhg-1* expressing cells act as posterior organizing centers, much in the same manner observed for polarizing region grafts (Saunders and Gasseling, 1968).

Curiously, we observed duplications of proximal skeletal elements such as the humerus, radius and ulna at a frequency of 65% in mid-distal border grafts (Fig. 8D; see Table 1), but never with anterior border grafts (Fig. 8C; see Table 1). To our knowledge, a strong correlation between graft location and duplication of proximal skeletal elements has not been previously noted, although previously reported results are consis-

Table 1. Skeletal element duplications induced by grafts of QT6 cells transfected with pCISHhg-1

Percentage of most posterior duplicated digit (n)				
Graft (n)	II	III	IV	Normal
Anterior hedgehog (29)	14% (4)	41% (12)	31% (9)	14% (4)
Mid-distal hedgehog (17)	17.5% (3)	65% (11)	0 (0)	17.5% (3)
β -galactosidase (11)	0	0	0	100% (11)
Posterior hedgehog (7)	0	0	0	100% (7)

Percentage of proximal element duplications* (n)				
Graft (n)	Radius	Ulna	Humerus	Normal
Anterior hedgehog (20)	0	0	0	100% (20)
Mid-distal hedgehog (17)	41% (7)	17.5% (3)	11.5% (2)	41% (7)
β -galactosidase (11)	0	0	0	100% (11)
Posterior hedgehog (7)	0	0	0	100% (7)

*A single specimen might contribute to more than one column.

Percentage of grafts that induced extra skeletal elements (n)		
Graft (n)	Duplications	Normal
Hedgehog (46)	87% (40)†	13% (6)
β -galactosidase (11)	0	100% (11)
Posterior hedgehog (7)	0	100% (7)

†38 specimens showed digit duplications.

QT6 cells transfected with either pCISHhg-1 or pCISLacZ were grafted to anterior, mid-distal or posterior borders of stage 20 chick limb buds. Embryos harvested at day 10 were fixed overnight in 10% formaldehyde, stained with Victoria Blue and cleared with methyl salicylate.

tent with our observation (see Discussion). The overall level of proximal or distal element duplications in all limbs receiving anterior or mid-distal border grafts of *Hhg-1*-expressing cells was 86.5% (Table 1). These percentages are similar to those reported by Riddle et al. (1993) following anterior grafts of cells infected with a retrovirus carrying *Shh*, a *hedgehog* family member in the chicken that probably corresponds to *Hhg-1*.

Proteolytic processing of the *Hhg-1* protein product

We have used affinity purified antibodies directed against epitopes from two distinct portions of the *Hhg-1* ORF (Fig. 9A) to confirm that *Hhg-1* encoded protein indeed is expressed in both systems where we have assayed for *Hhg-1* activity. As shown in the immunoblots of Fig. 9B, QT6 cells transfected with the pCISHhg-1 expression vector produce a polypeptide species of $\sim 45 \times 10^3 M_r$ which is detected by both N- and C-terminal specific antibodies in transfected cells (lanes 1 and 3, respectively). In addition, a $\sim 19 \times 10^3 M_r$ species is specifically detected by the N-terminal antibody while the C-terminal antibody specifically detects a $\sim 28 \times 10^3 M_r$ species. Neither the

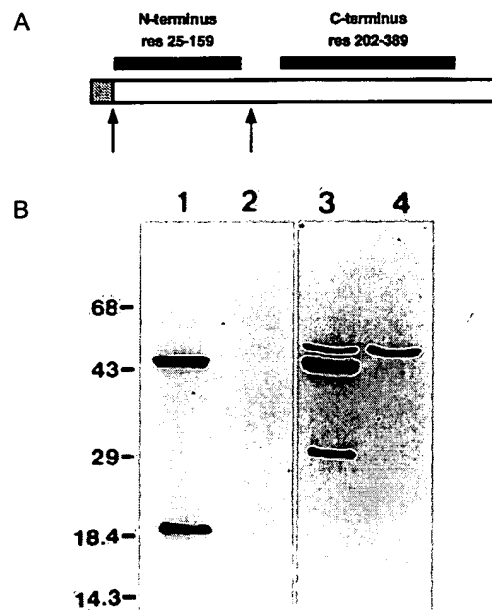


Fig. 9. Proteolytic processing of the *Hhg-1* protein. The filled boxes in A denote the portions of the *Hhg-1* ORF used to elicit antibodies, specific to the amino- and carboxy-terminal portions of the protein. The immunoblot in B illustrates the reactivity of amino-terminal (lanes 1 and 2) and carboxy-terminal (lanes 3 and 4) antibodies with species present in QT6 cells either transfected (lanes 1 and 3) or not transfected (lanes 2 and 4) with pCISHhg-1. Note the presence of a $\sim 45 \times 10^3 M_r$ transfection-dependent species detected by both antibodies. Each antibody also detects a single smaller species of $\sim 19 \times 10^3 M_r$ for the amino-terminal antibody and $\sim 28 \times 10^3 M_r$ for the carboxy terminal antibody. The slightly larger species detected in lanes 3 and 4 is not transfection dependent, but provides a control for the amount of protein loaded. The arrows in A denote a signal cleavage (following the shaded hydrophobic domain) and a proposed internal cleavage that can account for the observed species and their reactivities (see text).

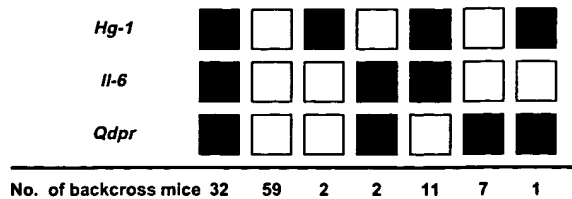


Fig. 10. Segregation of *Hhg-1* among proximal mouse chromosome 5 loci in [(C3H/HeJ-*gld* × *Mus spretus*)F₁ × C3H/HeJ-*gld*] interspecific backcross mice. Closed boxes represent the homozygous C3H pattern and open boxes the F₁ pattern. The number of mice with each haplotype is shown at the bottom of each column. The informative RFLV for *Hhg-1* is described in the text. The informative RFLVs for *Il-6* were generated by *TaqI* (C3H, 5.0 kb and 2.2 kb; *Mus spretus*, 8.6 kb and 1.9 kb) and for *Qdpr* were generated by *EcoRI* (C3H, 9.5 and 6.5 kb; *Mus spretus*, 8.2 kb).

large common species nor the smaller specific species are detected in untransfected cells (lanes 2 and 4). Essentially identical species were detected in protein extracts from heat shocked *Drosophila* embryos carrying the *hsHhg-1* construct, but not in extracts from unshocked embryos (D. T. C. and P. A. B., data not shown).

The arrows in Fig. 9A denote cleavages of the primary protein product that could account for *Hhg-1* species of the observed size. The first of these occurs at the *Hhg-1* signal sequence and is observed in a microsome-dependent fashion in *in vitro* translation reactions (J. J. Lee and P. A. B., unpublished data). The second internal cleavage is proposed as a simple possibility that can account for the observed polypeptide species and is similar to an internal cleavage that occurs in the *Drosophila hedgehog* protein precursor (J. J. Lee, S. C. Ekker and P. A. B., unpublished; see Discussion).

Chromosomal localization and fine mapping of *Hhg-1*

In order to determine the chromosomal location of the *Hhg-1* gene and to assess potential linkage with mouse developmental mutants, we analyzed a panel of DNA samples from an interspecific cross that has been characterized for over 500 genetic markers throughout the genome. The genetic markers included in this map span between 50 and 60 centi-Morgans (cMs) on each mouse autosome and on the X Chromosome (for examples see Saunders and Seldin, 1990; Watson et al., 1992). Initially, DNA from the two parental mice [C3H/HeJ-*gld* and (c3H/HeJ-*gld* × *Mus spretus*)F₁] were digested with various restriction endonucleases and hybridized with *Hhg-1* cDNA probe to determine restriction fragment length variants (RFLVs) thereby allowing haplotype analyses. Informative RFLVs were detected with *MspI* restricted DNAs: C3H/HeJ-*gld*, 13.0 kb; *Mus spretus*, 5.0 kb. Comparison of the haplotype distribution of the *Hhg-1* indicated that in 109 of the 114 meiotic events examined, the *Hhg-1* locus cosegregated with *Il-6* (Fig. 10), a locus previously mapped to proximal mouse Chromosome 5 (Mock et al., 1989; Kozak and Stephenson, 1993). The best gene order (Bishop, 1985) ± the standard deviation (Green, 1981) indicated the following gene order from proximal to distal: *Hhg-1* – 4.4 cM ± 1.9 cM – *Il-6* – 15.7 cM ± 3.5 cM – *Qdpr*.

DISCUSSION

Patterning functions of *Hhg-1*

The most remarkable feature of *Hhg-1* expression, which has also been noted for other closely related genes in multiple vertebrate species (Riddle et al., 1993; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994), is its occurrence in a number of embryonic tissues demonstrated to exert patterning influences on neighboring structures. The notochord and floor plate, for example, are capable of imposing ventral pattern upon the neural tube (reviewed in Jessell and Dodd, 1993), while the posterior margin of the vertebrate limb bud or polarizing region can function as a posterior organizing center when grafted to a developing limb. Grafting experiments also suggest that these organizing activities may be functionally related, since notochord and floor plate tissue can also function as posterior organizing centers when grafted to limb buds (Wagner et al., 1990).

Riddle et al. (1993) indeed showed that the chicken *Shh* gene encodes a product capable of imposing pattern upon developing chick limbs while Echelard et al. (1993) showed that ectopic expression of chicken *Shh* can induce inappropriate expression of ventral neural tube markers in the mouse; Krauss et al. (1993) also showed that ectopic expression of fish *shh* can induce inappropriate expression of ventral neural tube markers in fish embryos. Finally, Roelink et al. (1994) demonstrated that COS cells expressing the rat gene *vhh-1* can induce formation of floor plate and motor neurons when cocultured with lateral neural tube explants from rat.

The xenoplastic activities of *Hhg-1* described here represent the first direct assays of function for the mouse member of the *vhh-1* or *sonic* class of vertebrate *hh*-like sequences. Our results also demonstrate for the first time the activity of a mammalian *hh*-like gene in limb patterning. Consistent with the expression of *Hhg-1* in the posterior margin of mouse limb buds, polarizing activity previously has been identified in this location by grafting experiments (Tickle et al., 1976; Fallon and Crosby, 1977). In addition, preliminary results in the explant assay system suggest that the *Hhg-1* product can also induce floor plate formation in rat lateral neural tube (J. Dodd, D. T. C. and P. A. B., unpublished data). We thus conclude that the *Hhg-1* gene encodes patterning activities and that the expression of pattern of *Hhg-1* in the embryo could account, at least in part, for the patterning activities of specific tissues assayed by grafting experiments.

A noteworthy feature of our grafting operations was the high relative frequency of proximal skeletal element duplications in mid-distal grafts (65%) versus anterior grafts (0%). Although such a correlation between graft location and the occurrence of proximal duplications has not been previously noted, a cursory review of the literature from the first polarizing region grafts of Saunders and Gasseling (1968) onwards suggests that graft location indeed appears to operate as a determinant for formation of proximal element duplications. More recently, Riddle et al. (1993) reported proximal element duplications induced by anterior grafts of cells expressing the chicken *Shh* gene. In both cases of proximal element duplication depicted, however, the digit sequence indicated a location sufficiently posterior to allow formation of at least one digit anterior to the graft, thus reinforcing the correlation between proximal

element duplications and a more posterior graft location (at least as far posterior as mid-distal). The significance of this observation remains to be investigated.

Genetic linkage of *Hhg-1*

Our segregation analysis of *Hhg-1* indicates a localization to the proximal region of mouse chromosome 5. Given the ability of *Hhg-1* to function in limb patterning, our attention was drawn to two mutations affecting limb development that also map to this region of mouse chromosome 5. One, *Hm* (hammertoe), is a semidominant mutation causing failure of normal programmed cell death in the webbing between toes during development, resulting in the formation of contractures in the second phalanx of all four limbs in the adult. This phenotype is somewhat more pronounced in homozygotes, which nevertheless remain viable and fertile. The second, *Hx* (Hemimelic extra toes), is also a dominant mutation associated with shortening or complete absence of tibia and talus in the hindlimbs and shortening of the radius in the forelimbs; in addition, metatarsals or metacarpals are duplicated giving a total of seven to eight digits per paw instead of the normal five (Dickie, 1968; Knudsen and Kochhar, 1981). The homozygous phenotype of *Hx* is an uncharacterized embryonic lethality (Knudsen and Kochhar, 1981). *Hx* and *Hm* are very closely linked but separate mutations, having been observed to recombine in only 1 of 3664 offspring from *trans*-heterozygous parents (Sweet, 1982). In addition to these mutations in the mouse, the syntenic region of human chromosome 7q has also been identified as the genetic locus for several developmental anomalies involving polydactyly (Tsukurov et al., 1994; Heutink et al., 1994).

In order to investigate the possibility that one or both of the mouse mutations affect the *Hhg-1* gene, we examined by Southern blotting the restriction pattern of DNA from both of these mutants. Using nine different restriction endonucleases for *Hx* and for *Hm*, we detected no differences between parental and mutant DNA (data not shown). Since the *Hx* phenotype suggests a defect in early limb patterning, as might be expected from a mutation in the *Hhg-1* gene, we attempted to discover alterations in *Hhg-1* coding sequences in the *Hx* mutant. Because only heterozygous *Hx* mutant DNA was available (from Jackson Labs), our conclusions depend upon analysis of multiple independently isolated clones. We examined eleven, fourteen and eight independent clones from the coding portions of exons one, two and three, respectively, without detecting any differences from wild type (see Materials and Methods). Since the clones for sequence determination were derived using the polymerase chain reaction, it is possible that a deletion(s) at the *Hhg-1* locus might have prevented amplification of the mutant allele. Given the uncertainty inherent in sampling from heterozygous DNA, it is also formally possible, although highly unlikely, that we could have missed a coding difference in the *Hhg-1* gene of *Hx* mutants.

In the absence of *Hx*- or *Hm*-associated alterations in *Hhg-1* coding sequence, another possibility to consider is that the *Hx* or *Hm* phenotypes could result from a mutation in *cis*-acting regulatory regions of *Hhg-1*, causing either a reduction of *Hhg-1* expression or inappropriate spatial localization of expression. Given our Southern blotting results, such a lesion could lie near the *Hhg-1* gene only if it is sufficiently subtle to escape detection by Southern blotting with our cDNA probe;

alternatively, a *Hhg-1* regulatory lesion may have escaped detection because of a location distant from sequences represented within the *Hhg-1* cDNA.

With regard to potential mechanisms underlying genetic dominance for such a regulatory mutation, the dominant limb deformity mutation *Xt* (extra toes) may be informative. Like *Hx*, *Xt* causes polydactyly and is lethal when homozygous; mutations affect the gene *GLI3*, which encodes a zinc finger transcription factor. At least one allele of *Xt* appears to act simply by disrupting transcription of *GLI3* (Schimmang et al., 1992), and thus, the genetic dominance of mutations at this locus is probably due to haploinsufficiency. The *GLI3* gene is also interesting in that its close *Drosophila* relative, the gene *cubitus-interruptus*^{Dominant} (*ci^D*), functions downstream in the *hedgehog* signaling pathway (Forbes et al., 1993). If the *GLI3* gene similarly functions downstream of *Hhg-1* in the mouse, and given that *GLI3* function is haploinsufficient, it would not be surprising to find that partial loss of *Hhg-1* expression caused by a regulatory mutation might also have a dominant phenotype. Interestingly, a human polysyndactyly disease that maps to a region of human chromosome 7 syntenic to the region containing *Hhg-1* and *Hm* and *Hx* is also inherited in a dominant fashion (Tsukurov et al., 1994; Heutink et al., 1994). Alternatives to haploinsufficiency are that a regulatory mutation might cause *Hhg-1* mis-expression or that *Hx* and *Hm* are unrelated to *Hhg-1*.

Duplication and divergence of the *hedgehog* gene family in vertebrates

Our PCR-based search for vertebrate *hedgehog* homologues yielded the three distinct mouse and two distinct human sequences reported here, and five sequences each from the zebrafish *Brachydanio rerio* and the toad *Xenopus laevis* (S. C. Ekker, J. J. Lee, D.v.K. and P. A. B., unpublished data). In contrast, none of the invertebrate species to which our PCR-based method was applied yielded more than a single distinct *hh*-like sequence. For example, using various combinations of degenerate primers from conserved regions, eighteen independent *Drosophila melanogaster* clones identical to *hh* were isolated without encountering any diverged *hh*-like sequences. It is not yet possible to estimate accurately the total number of distinct vertebrate *hh*-like genes. The occurrence of multiple *hh*-like sequences in vertebrates but not invertebrates nevertheless suggests that at some point during evolution of the vertebrate lineage repeated duplication and divergence of a single ancestral *hedgehog* gene occurred, as has been proposed for the origin of multiple vertebrate HOM-C gene clusters from a single ancestral cluster (Schubert et al., 1993).

Broad evolutionary conservation of *hedgehog* protein function and proteolytic processing

The evolutionary conservation of *hh* extends beyond sequence to include function, as demonstrated by the ability of *Hhg-1* to encode a signal capable of inducing expansion of the *wingless* stripe of expression in *Drosophila* embryos. Similar results using a *hh*-like gene isolated from zebrafish were also reported by Krauss et al. (1993). If the proposal, based on genetic arguments, that the gene *patched* (*ptc*) encodes a *hh* receptor in *Drosophila* is correct (Ingham et al., 1991), the functional conservation of vertebrate *hedgehog* signals would suggest that *ptc*-like sequences and function should also be conserved in

vertebrates. With regard to the identity of a *hh* receptor, however, we observed that both *hh* and *Hhg-1* can induce broadening of the *wingless* stripe when ectopically expressed at the retracted germ band stage of *Drosophila* development. By this stage, the initially broad stripe of *ptc* mRNA and protein expression has split into two thinner stripes per segment by loss of expression from the cells in the middle of the broad stripe (Taylor et al., 1993). Expanded *wingless* expression in response to *hh* thus is occurring in interstripe cells that in normal embryos no longer express the *ptc* protein. Ingham (1993) has reported that *ptc* expression in these interstripe cells is also induced by ectopic *hedgehog*, but it is not known whether *ptc* induction in the interstripe precedes or follows *wg* induction in the interstripe cells. Whatever the sequence of induction, novel expression of *ptc* or *wg* represents a response to *hh* protein in interstripe cells, which do not express the *ptc* protein, thus suggesting that *ptc* does not encode the *hh* receptor, or at least not the only receptor.

The occurrence of multiple *Hhg-1* polypeptide species in *Drosophila* embryos as well as in avian cells raises a question as to the role of proteolytic processing in *hedgehog* protein function. We believe that the N- and C-terminally derived forms of the *Hhg-1* protein bear a product/precursor relationship to the larger form because the relative molecular masses of the smaller products sum to yield approximately the relative molecular mass of the larger product, and they could therefore be derived by a single internal cleavage as shown in the model in Fig. 9A. The location of this internal cleavage coincides approximately with an intron/exon boundary and with a sharp demarcation in the degree of sequence conservation (see Results). In addition, these smaller forms resemble smaller forms of the *hh* protein observed in *Drosophila* (Tabata and Kornberg, 1994; J. J. Lee and P. A. B., unpublished data), where an internal cleavage occurs and appears to be required for the *hh* signaling function (J. J. Lee, S. C. Ekker, D. P. von K. and P. A. B., unpublished data).

The existence of two distinct stable products derived from a single larger precursor may provide a clue to the apparent dual nature of *hedgehog* gene action in several developmental systems. In the *Drosophila* embryo, for example, the restriction of *wingless* gene expression to a narrow stripe within each segment is dependent upon the short-range nature of a *hedgehog* signaling activity (see above; Ingham, 1993); in contrast, the influence of a later-acting *hh*-encoded activity extends across most of the segment in imposing pattern upon the dorsal cuticle (Heemskerk and DiNardo, 1994). Similarly in ventral neural tube patterning, induction of floor plate occurs at short range and depends upon direct contact with notochord, floor plate, or COS cells expressing *vhh-1* (Placzek et al., 1993; Roelink et al., 1994). COS cells expressing *vhh-1* also have motor neuron inducing activity (Roelink et al., 1994). This latter activity is found in diffusible form in supernatants from notochord and floor plate cultures (Yamada et al., 1993), although it is not yet clear that *vhh-1* directly encodes the diffusible activity. Long- and short-range *hedgehog* activities have not been definitively identified in the context of limb patterning, but such activities have been extensively discussed; dual modes of *hedgehog* action thus may yet emerge from studies of such apparently distinct activities as influences upon the apical ectodermal ridge and anterior/posterior patterning of the developing limb.

An alternative would be that only one of the smaller *hedgehog* protein species is biologically active, with the apparent dual nature of *hedgehog* action deriving from secondary effects. For example, restricted diffusion for the primary active species could produce apparent long-range effects by inducing expression of another diffusible molecule. Similarly, a diffusible or primarily long-range *hedgehog* signal could yield apparent short-range effects through threshold-dependent responses of target cells. To resolve these questions, the structures and embryonic localizations of the *hedgehog*-encoded proteins must be determined and their patterning activities assayed. At another level, a true understanding of the functional roles of vertebrate *hedgehog* proteins requires a demonstration that patterning functions in vertebrate embryos actually are executed by the products of this class of genes; this would best be achieved through specific inactivation of *hedgehog* gene products by genetic or other means.

We gratefully acknowledge J. Gearhart, S.-J. Lee, C. Moore, C. Mjaatvedt and T. Huynh for instruction in handling of mice, manipulation of embryos, and advice on in situ hybridisation. We also thank A. Lanahan, J. Williams and S.-J. Lee for mouse cDNA libraries and RNA blots. K. Young, M. Claudia, D. Sullivan, J. Kassis, S. Brown, R. Denell, G. Aisenberg and A. Cameron generously provided genomic DNA and libraries from various species, and K. Young graciously assisted in the isolation and characterization of the mosquito and mouse sequences. We are indebted to N. Patel for equipment and help with dark field microscopy. We also thank J. Dodd, J. J. Lee and S. C. Ekker, for sharing unpublished data, and S. C. Ekker for help with figures. T. Vogt and S.-J. Lee provided useful comments on the manuscript. This work was supported by the Howard Hughes Medical Institute, an NIH MSTP award to D. T. C., NIH awards HD20743 and ST32GM07507 to J. F. F. and A. L., and NIH award HG00734 to M. F. S.

REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1993). *Current Protocols in Molecular Biology*. New York: Greene Publishing Associates and Wiley-Interscience.
- Baker, N. E. (1988). Embryonic and imaginal requirements for *wingless*, a segment polarity gene in *Drosophila*. *Dev. Biol.* 125, 96-108.
- Bejsovec, A. and Martinez-Arias, A. (1991). Roles of *wingless* in patterning the larval epidermis of *Drosophila*. *Development* 113, 471-485.
- Bejsovec, A. and Wieschaus, E. (1993). Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. *Development* 119, 501-517.
- Bishop, D. T. (1985). The information content of phase-known matings for ordering genetic loci. *Genet. Epidemiol.* 2, 349-361.
- Burnette, W. N. (1981). Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated Protein A. *Anal. Biochem.* 112, 195-203.
- Chen, C. and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Molecular & Cellular Biology* 7, 2745-2752.
- Dickie, M. M. (1968). *Mouse News Lett.* 38, 24.
- DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J. A. and O'Farrell, P. H. (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* 332, 604-609.
- Dougan, S. and DiNardo, S. (1992). *Drosophila wingless* generates cell type diversity among *engrailed* expressing cells. *Nature* 360, 347-50.
- Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75, 1417-1430.
- Fallon, J. F. and Crosby, G. M. (1977). Polarizing zone activity in limb buds

- of amniotes. In *Vertebrate Limb and Somite Morphogenesis*. (ed. D. A. Ede, J. R. Hinchliffe and M. Balls), pp 55-70. Cambridge: England.
- Feinberg, A. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.
- Ferguson, E. L. and Anderson, K. V. (1992). Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71**, 451-461.
- Forbes, A. J., Nakano, Y., Taylor, A. M. and Ingham, P. W. (1993). Genetic analysis of *hedgehog* signalling in the *Drosophila* embryo. *Development* **193** Supplement, 115-124.
- Gorman, C. (1985). High efficiency gene transfer into mammalian cells. In *DNA cloning*. (ed. D. M. Glover), pp 143. Oxford.
- Green, E. L. (1981). Linkage, recombination and mapping. In *Genetics and Probability in Animal Breeding Experiments*. (ed. E. Green), pp 77-113. New York.
- Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Publications.
- Heberlein, U., Wolff, T. and Rubin, G. M. (1993). The TGF beta homolog *dpp* and the segment polarity gene *hedgehog* are required for propagation of a morphogenetic wave in the *Drosophila* retina. *Cell* **75**, 913-926.
- Heemskerk, J. and DiNardo, S. (1994). *Drosophila hedgehog* acts as a morphogen in cellular patterning. *Cell* **76**, 449-460.
- Heutink, P., Zguricas, J., Oosterhout, L. v., Breedveld, G. J., Testers, L., Sandkuijl, L. A., Snijders, P. J. L. M., Weissenbach, J., Lindhout, D., Hovius, S. E. R. and Oostra, B. A. (1994). The gene for triphalangeal thumb maps to the subtelomeric region of chromosome 7q. *Nature Genetics* **6**, 287-292.
- Ingham, P. W. (1993). Localized *hedgehog* activity controls spatial limits of wingless transcription in the *Drosophila* embryo. *Nature* **366**, 560-562.
- Ingham, P. W., Taylor, A. M. and Nakano, Y. (1991). Role of the *Drosophila patched* gene in positional signalling. *Nature* **353**, 184-187.
- Jessell, T. M. and Dodd, J. (1993). Control of neural cell identity and pattern by notochord and floor plate signals. In *Cell-Cell Signaling in Vertebrate Development*. (ed. E. J. Robertson, F. R. Maxfield and H. J. Vogel), pp 139-155. San Diego.
- Knudsen, T. B. and Kochhar, D. M. (1981). The role of morphogenetic cell death during abnormal limb-bud outgrowth in mice heterozygous for the dominant mutation *Hemimelia-extra toe* (*Hmx*). *J. Embryol. Exp. Morphol.* **65** (Supplement), 289-307.
- Kozak, C. A. and Stephenson, D. A. (1993). Mouse chromosome 5. *Mammalian Genome* **4**, s72-87.
- Krauss, S., Concordet, J.-P. and Ingham, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lee, J. J., von Kessler, D. P., Park, S. and Beachy, P. A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene *hedgehog*. *Cell* **71**, 33-50.
- Lee, S. J. (1990). Identification of a novel member (GDF-1) of the transforming growth factor- β superfamily. *Mol. Endocrinol.* **4**, 1034-1040.
- Lockyer, J., Cook, R. G., Milatien, S., Kaufman, S., Woo, S. L. C. and Ledley, F. D. (1987). Structure and expression of human dihydropteridine reductase. *Proc. Natl. Acad. Sci. USA* **84**, 3329-3333.
- Ma, C., Zhou, Y., Beachy, P. A. and Moses, K. (1993). The segment polarity gene *hedgehog* is required for progression of the morphogenetic furrow in the developing *Drosophila* eye. *Cell* **75**, 927-938.
- Martinez Arias, A., Baker, N. E. and Ingham, P. W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**, 157-170.
- Mock, B. A., Nordan, R. P., Justice, M. J., Kozak, C., Jenkins, N. A., Copeland, N. G., Clark, S. C., Wong, G. G. and Rudnikoff, S. (1989). The murine *Il-6* gene maps to the proximal region of Chromosome 5. *Journal of Immunology* **142**, 1372-1376.
- Mohler, J. (1988). Requirements for *hedgehog*, a segmental polarity gene, in patterning larval and adult cuticle of *Drosophila*. *Genetics* **120**, 1061-1072.
- Mohler, J. and Vani, K. (1992). Molecular organization and embryonic expression of the *hedgehog* gene involved in cell-cell communication in segmental patterning in *Drosophila*. *Development* **115**, 957-971.
- Moscovici, C., Moscovici, M. G., Jimenez, H., Lai, M. M., Hayman, M. J. and Vogt, P. K. (1977). Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. *Cell* **11**, 95-103.
- Nusse, R. and Varmus, H. E. (1992). *Wnt* genes. *Cell* **69**, 1073-1087.
- Peifer, M. and Bejsovec, A. (1992). Knowing your neighbors: cell interactions determine intrasegmental patterning in *Drosophila*. *Trends in Genetics* **8**, 243-249.
- Placzek, M., Jessell, T. M., and Dodd, J. (1993). Induction of floor plate differentiation by contact-dependent homeogenetic signals. *Development* **117**, 205-218.
- Riddle, D. R., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.
- Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987). The *Drosophila* homolog of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* **50**, 649-657.
- Riley, B. B., Savage, M. P., Simandl, B. K., Olwin, B. B. and Fallon, J. F. (1993). Retroviral expression of FGF-2 (bFGF) affects patterning in chick limb bud. *Development* **118**, 95-104.
- Roelink, H., Augsburger, A., Heemskerk, J., Korch, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, J. (1994). Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of *hedgehog* expressed by the notochord. *Cell* **76**, 761-775.
- Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edition Cold Spring Harbor, Cold Spring Harbor Laboratory Publications.
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- Saunders, A. M. and Seldin, M. F. (1990). A molecular genetic linkage map of mouse chromosome 7. *Genomics* **8**, 524-535.
- Saunders, J. W. and Gasseling, M. (1968). Ectodermal-mesenchymal interaction in the origin of limb symmetry. In *Epithelial-Mesenchymal Interaction*. (ed. R. Fleischmayer and R. E. Billingham), pp 78-97. Baltimore.
- Schimmgang, T., Lemaistre, M., Vortkamp, A. and Ruther, U. (1992). Expression of the zinc finger gene *Gli3* is affected in the morphogenetic mouse mutant *extra-toes* (*Xt*). *Development* **116**, 799-804.
- Schubert, F. R., Nieselt-Struwe, K. and Gruss, P. (1993). The Antennapedia-type homeobox genes have evolved from three precursors separated early in metazoan evolution. *Proc. Natl. Acad. Sci. USA* **90**, 143-147.
- Seldin, M. F., Morse, H. C., Reeves, J. P., Scribner, J. P., LeBoeuf, R. C. and Steinber, A. D. (1988). Genetic analysis of autoimmune *gld* mice I. Identification of a restriction fragment length polymorphism closely linked to the *gld* mutation within a conserved linkage group. *J. Exp. Med.* **167**, 688-693.
- Sweet, H. O. (1982). *Mouse News Lett.* **66**, 66.
- Tabata, T., Eaton, S. and Kornberg, T. (1992). The *Drosophila hedgehog* gene is expressed specifically in posterior compartment cells and is a target of *engrailed* regulation. *Genes Dev.* **6**, 2635-2645.
- Tabata, T. and Kornberg, T. B. (1994). *Hedgehog* is a signaling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76**, 89-102.
- Tashiro, S., Michiue, T., Higashijima, S., Zenko, S., Ishimaru, S., Takahashi, F., Orihara, M., Kojima, T. and Saigo, K. (1993). Structure and expression of *hedgehog*, a *Drosophila* segment polarity gene required for cell-cell communication. *Gene* **124**, 183-189.
- Tautz, D. and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in the *Drosophila* embryo reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.
- Taylor, A. M., Nakano, Y., Mohler, J. and Ingham, P. W. (1993). Contrasting distributions of *patched* and *hedgehog* proteins in the *Drosophila* embryo. *Mech. Dev.* **42**, 89-96.
- Thummel, C. S., Boulet, A. M. and Lipshitz, H. D. (1988). Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. *Gene* **74**, 445-456.
- Tickle, C., Shellswell, G., Crawley, A. and Wolpert, L. (1976). Positional signalling by mouse limb polarising region in the chick wing bud. *Nature* **259**, 396-397.
- Tsukurov, O., Boehmer, A., Flynn, J., Nicolai, J.-P., Hamel, B. C. J., Traill, S., Zaleske, D., Mankin, H. J., Yeon, H., Ho, C., Tabin, C., Seidman, J. G. and Seidman, C. (1994). A complex bilateral polysyndactyly disease locus maps to chromosome 7q36. *Nature Genetics* **6**, 282-286.
- von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucl. Acids Res.* **14**, 4683-4690.
- Wagner, M., Thaller, C., Jessell, T. and Eichele, G. (1990). Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* **345**, 819-822.

- Watson, M. L., D'Eustachio, P. D., Mock, B. A., Steinberg, A. D., Morse, H. C. I., Oakey, R. J., Howard, T. A., Rochelle, J. M. and Seldin, M. F. (1992). A linkage map of mouse chromosome 1 using an interspecific cross segregating for the *gld* autoimmunity mutation. *Mammalian Genome* **2**, 158-171.
- Wieschaus, E. and Nusslein-Volhard, C. (1986). Looking at embryos. In *Drosophila: A Practical Approach*. (ed. D. B. Roberts), pp 199-227. Oxford: England.
- Wilkinson, D. G. (1992). Whole mount *in situ* hybridization of vertebrate embryos. In *In situ hybridization: A practical approach*. (ed. D. G. Wilkinson), pp 75-83. Oxford.
- Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology* **25**, 1-47.
- Yamada, T., Pfaff, S. L., Edlund, T., and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73**, 673-686.

(Accepted 12 August 1994)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☒ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.